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High-performance liquid chromatographic assay for the determination of sulfadoxine and *N*-acetyl sulfadoxine in plasma from patients infected with sensitive and resistant *Plasmodium falciparum* malaria

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Abstract

A reversed-phase high-performance liquid chromatographic method using a mobile phase of acetonitrile–methanol–trifluoroacetic acid–water (16.1:7.2:0.1:76.6, v/v/v/v) at a flow rate of 1.0 ml min^{-1} on a LiChrospher™ RP-18 column with UV (254 nm) detection has been developed for the separation of sulfadoxine and its metabolite *N*-acetyl sulfadoxine in plasma. No interferences due to endogenous compounds or common antimalarial drugs were noticed. The limit of detection for sulfadoxine and *N*-acetyl sulfadoxine was 0.01 μg ml^{-1} with a signal-to-noise ratio of 5:1 while the limit of quantification was 2.5 μg ml^{-1}. Intra-day mean relative standard deviations (RSD's) for sulfadoxine and *N*-acetyl sulfadoxine were 2.6 and 2.8%, respectively, while mean inter-day RSD's for sulfadoxine and *N*-acetyl sulfadoxine were 2.4 and 2.8%, respectively. Extraction recoveries averaged 90.6% for sulfadoxine and 86.9% for *N*-acetyl sulfadoxine. The method was applied for the assay of sulfadoxine and its metabolite *N*-acetyl sulfadoxine in plasma from *Plasmodium falciparum* malaria patients. Mean plasma sulfadoxine concentrations on day 2 (51 h) from samples collected from sensitive and resistant *P. falciparum* patients treated with three tablets of Fansidar™ were 62.8 and 60.5 μg ml^{-1}, respectively. Mean ratio of *N*-acetyl sulfadoxine to sulfadoxine was 9.1% for responders and 13.9% for non-responders which revealed that higher amounts of the metabolite *N*-acetyl sulfadoxine were present in non-responders. The method described should find an application in the therapeutic monitoring of malaria patients.

Keywords: HPLC; Sulfadoxine; *N*-acetyl sulfadoxine; Malaria cases

1. Introduction

Sulfadoxine–pyrimethamine (Fansidar™) has been used extensively against chloroquine resistant *Plasmodium falciparum*. Recently sulfadoxine–pyrimethamine assumed greater significance because of its possible role in combination therapy with artemisinin derivatives [1]. Determination of antimalarial drug concentrations during treatment has been proposed by World Health Organization (WHO) for the definition and identification of drug resistance. Many HPLC methods have been developed to determine the concentration of sulfadoxine [2–7] and its metabolite *N*-acetyl sulfadoxine in human plasma [2,4,8] from healthy volunteers and in capillary blood dried on filter paper [9,10]. Recently Sinnaeve et al. [11] reported a liquid chromatographic–mass spectrometric assay for the determination of sulfadoxine in human plasma while Bhoir et al. [12] used packed column supercritical fluid chromatography for monitoring sulfadoxine in the same matrix. While some information is available concerning sulfadoxine concentrations in plasma from malaria patients [13–16] and the pharmacokinetics of sulfadoxine have been studied in this group [16,17], many assays fail to monitor *N*-acetyl sulfadoxine, a primary metabolite in plasma that is important in assessing the outcome of treatment. Therapeutic failure during Fansidar™ treatment may be due to rapid acetylation of sulfadoxine. Sarikabhuti et al. [14] have suggested that non-responders metabolize sulfadoxine faster than

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responders. However, they used a relatively unselective spectrophotometric assay [18] to determine the concentrations of sulfadoxine and its metabolite *N*-acetyl sulfadoxine.

We describe here a simple and specific reversed-phase HPLC method for the simultaneous measurement of sulfadoxine and its metabolite *N*-acetyl sulfadoxine in human plasma and have applied it to the monitoring of plasma concentrations of both compounds in patients with drug-sensitive and drug-resistant *P. falciparum*.

2. Experimental

2.1. Reagents and sample preparation

HPLC grade acetonitrile and methanol were purchased from Ranbaxy Fine Chemicals, Delhi, India. Water was de-ionized and triple distilled. All other chemicals were of analytical grade and were used without further purification. Sulfadoxine, *N*-acetyl sulfadoxine and sulfamethoxazole (internal standard) were supplied by Roche Products Ltd., Bombay, India and their purity was ≥99%. Their structures are given in Fig. 1. Stock solutions of sulfadoxine, *N*-acetyl sulfadoxine and sulfamethoxazole (5 mg ml^{-1}) were prepared in methanol and stored at 4 °C. Intermediate and working standard solutions covering the concentration range reported earlier [13,19] were prepared by dilution of the stock solutions with methanol and were stored at 4 °C. Standards and quality control samples were prepared from the same stock solution. A phosphate buffer solution (12 mM; pH 3.40) was prepared by adding 0.1 ml of acetic acid to 9.9 ml of $K_2HPO_4{\cdot}3H_2O$ solution (0.273 g of di-potassium hydrogen phosphate-3 hydrate dissolved in 100 ml distilled water).

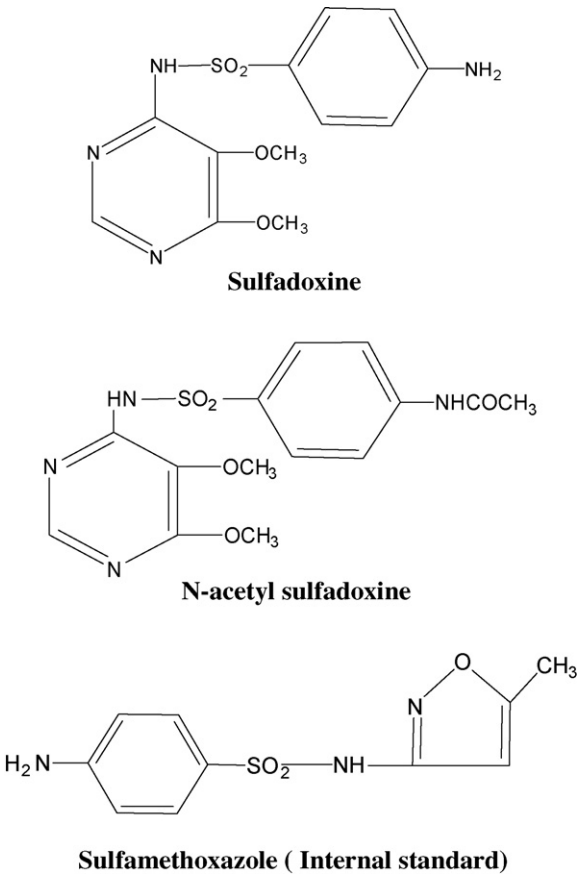

Fig. 1. Structures of sulfadoxine, *N*-acetyl sulfadoxine and sulfamethoxazole (internal standard).

2.2. Apparatus and HPLC conditions

A HPLC system consisting of a Waters 510 pump, 486 multi-wavelength UV detector (Waters Assoc. Milford, MA, USA) operated at 254 nm and 0.1 absorbance units full scale (AUFS), a Rheodyne^{TM} injector (Model 7125, Cotati, CA, USA) and a Shimadzu C-R8A Chromatopak data processor (Shimadzu Corporation, Kyoto, Japan) was used for analysis. A LiChrospher^{TM} RP-18 (E. Merck, Darmstadt, Germany) reversed-phase column (4 mm × 250 mm; particle size 5 μm) was used for the separation. The mobile phase consisted of acetonitrile–methanol–trifluoroacetic acid–water (16.1:7.2:0.1:76.6, v/v/v/v) and was delivered at a flow rate of 1.0 ml min^{-1} at ambient temperature. The mobile phase was filtered and degassed by ultra-sonication (Decon^{TM} FS 100, Hove, UK) before use.

2.3. Sample extraction

Extraction of sulfadoxine and its metabolite *N*-acetyl sulfadoxine was performed as described earlier [19]. Briefly, to plasma (0.5 ml) in a screw capped glass tube, were added sulfamethoxazole as internal standard (100 μl; 50 μg ml^{-1}), 0.5 ml of distilled water, phosphate buffer (100 μl; pH 3.40) and 1,2-dichloroethane (6 ml). The tube was agitated for 20 min on an orbital mixer (Denley; Billingshurst, UK) and centrifuged at 1000 × *g* for 15 min to separate the phases. The organic phase was transferred to a clean glass tube and reduced to dryness at 60 °C in a vortex evaporator. The residue was re-dissolved in mobile phase (50 μl) and 10 μl of the solution was injected on to the instrument for analysis.

2.4. Calibration

The linearity of the method was assessed by preparing calibration curves from plasma samples (0.5 ml) spiked with known concentrations of sulfadoxine and *N*-acetyl sulfadoxine (2.5, 10, 25, 50 and 100 μg ml^{-1}) and a blank sample with out internal standard. These values were selected based on an expected range of plasma concentration reported in Refs. [13,19]. Sulfamethoxazole (internal standard) concentration was 50 μg ml^{-1}. Unweighted linear least squares regression was used to assess the calibration curves and to determine correlation coefficient. Peak area ratio (drug:internal standard) was used for calibration. Calibration curves were generated using a statistical software Curve Expert 1.3 and calibration equation relating *y* (Drug/IS; peak area ratio) to × (Concentration μg ml^{-1}) and coefficient of the determination and % error were calculated.

2.5. Quality control samples

Quality control samples were prepared at five levels by addition of known amounts of sulfadoxine and *N*-acetyl sulfadoxine to drug-free plasma. These were 2.5 μg ml^{-1} (lower limit of quantification), 10 μg ml^{-1} (low), 25 μg ml^{-1}, 50 μg ml^{-1} (medium), and 100 μg ml^{-1} (high). Quality control samples were stored at −20 °C until analysis.

2.6. Method validation

2.6.1. Specificity

Retention times of other antimalarial compounds routinely used for the treatment of malaria were investigated in order to check for any interference. These were sulfalene, amodiaquine, dapsone, quinine, primaquine, pyrimethamine and chloroquine. To investigate potential endogenous interference, drug-free plasma samples from 15 human volunteers were analyzed.

2.6.2. Lower limit of detection (LLD) and quantification (LLQ)

The lower limit of detection for each compound was stated to be the minimum amount detectable by UV at 254 nm with signal-to-noise ratio of 5:1. The limit of quantification was defined as the lowest concentration on the calibration curve which could be measured with an intra-assay precision and accuracy <15% [8]. LLQ may be extrapolated if not fitted in the range of calibration curve.

2.6.3. Recovery

Recovery of sulfadoxine and *N*-acetyl sulfadoxine was determined by comparing of 10 preparations at each of the quality control samples concentrations of 2.5 (LLQ), 10, 25, 50 and 100 μg ml^{-1} to the response of pure authentic standards. Extraction recovery of sulfamethoxazole (internal standard) was determined at 50 μg ml^{-1}.

2.6.4. Precision and accuracy

Estimates of inter- and intra-assay precision were obtained by replicate assays of samples from the pools of spiked plasma at 2.5, 10, 25, 50 and 100 μg ml^{-1} of sulfadoxine and *N*-acetyl sulfadoxine.

The intra-assay precision was assessed from 10 plasma samples of each concentration stated above while inter-assay precision was determined by analysis over a period of 10 days of the same spiked plasma samples. Intra-assay and inter-assay precision for sulfamethoxazole was determined at 50 μg ml^{-1}. The relative standard deviations (RSD's) of the estimated concentrations were determined and used for the assessment of precision. Accuracy was determined as the percentage difference between the amount of drug added and the amount of drug measured. Quality control plasma supplemented with 2.5 (LLQ), 10, 25, 50 and 100 μg ml^{-1} of sulfadoxine and *N*-acetyl sulfadoxine and 50 μg ml^{-1} internal standard were prepared and stored at −20 °C. These samples were analyzed with the test samples. The criteria for acceptance were that 80% of the standard calibration samples should fall within ±15% of the mean value.

2.6.5. Stability

To test the short and long-term stability of sulfadoxine and *N*-acetyl sulfadoxine, two quality control samples one low (10 μg ml^{-1} of plasma) and one high (100 μg ml^{-1} of plasma) along with sulfamethoxazole (50 μg ml^{-1} of plasma) were stored under different conditions: at room temperature for 8 h and at −20 °C for 45 days. Stock solution stability was evaluated at 4 °C and at room temperature for 6 h. Freeze–thaw stability was also evaluated at 10 and 100 μg ml^{-1} concentrations. These samples were stored at −20 °C. Three samples were assayed on day 1, with another three thawed to room temperature and re-frozen. The cycle was repeated twice and a further analysis performed. The compounds were considered stable if assay variation was <10%.

2.6.6. Reinjection reproducibility

Reinjection reproducibility of the method was assessed by injecting 10 μl of sulfamethoxazole (internal standard; 50 μg ml^{-1}). Forty plasma extracted samples were injected and the sulfamethoxazole peak area of each injection was compared with the mean value. Five samples each of sulfadoxine (50 μg ml^{-1}) and *N*-acetyl sulfadoxine (50 μg ml^{-1}) were also evaluated for reinjection reproducibility.

2.7. Subjects

Infection of 52 *P. falciparum* malaria patients (age range 20–40 years) was confirmed by microscopic examination during the months of October–December 2005. Three tablets of Fansidar^{TM} (each consisting of 500 mg sulfadoxine and 25 mg pyrimethamine) were given to each individual as a single dose regimen schedule after establishing that there was no history of other drug intake and there was no known allergy to sulfonamides. Each patient was followed for 28 days to monitor *in vivo* sensitivity as per customary WHO procedures. Intravenous blood (2.0 ml) was drawn from each patient on day 2 (51 h) into a sterilized glass tube and centrifuged at 1000 × *g* for 15 min to separate plasma and blood cells. Heparin was used as an anticoagulant. Plasma was stored at −20 °C until analyzed.

3. Results and discussion

HPLC separation of sulfadoxine, *N*-acetyl sulfadoxine and sulfamethoxazole (internal standard) in plasma was accomplished by using a modification of the mobile phase of an earlier HPLC method used for the determination of sulfadoxine [13]. Essentially, trifluoroacetic acid (TFA) was used as an ion pairing reagent in the mobile phase acetonitrile–methanol–trifluoroacetic acid–water (16.1:7.2:0.1:76.6, v/v/v/v) for the separation of sulfadoxine, *N*-acetyl sulfadoxine and internal standard instead of perchloric acid, used in the earlier method, in a mobile phase of acetonitrile–methanol 1 M perchloric acid–water (22.2:6.7:0.6:70.5, v/v/v/v). The mobile phase was delivered

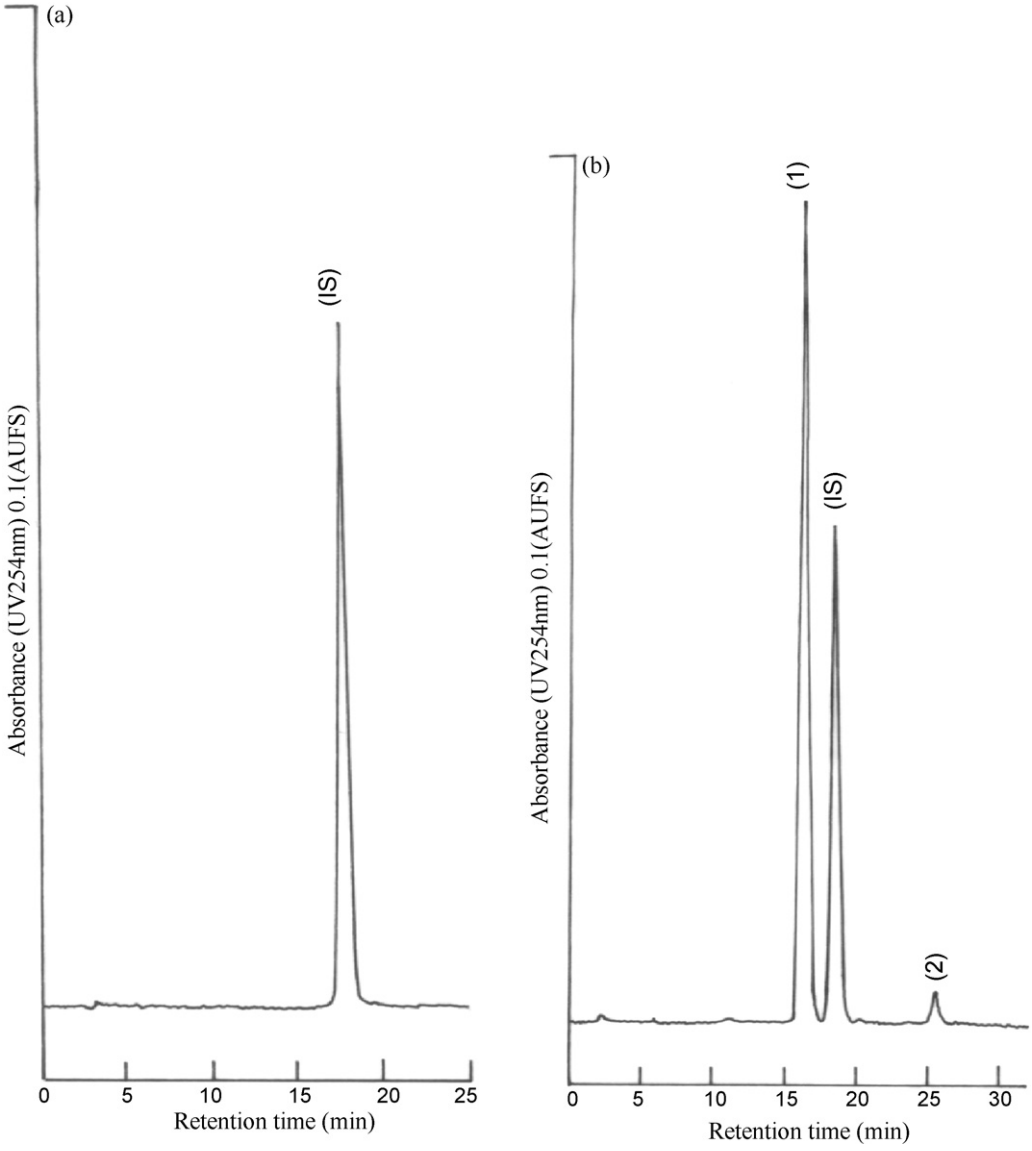

Fig. 2. (a) HPLC profile of an extracted blank plasma sample spiked with an internal standard. Peak IS; sulfamethoxazole (internal standard). (b) Chromatographic behavior of an extract of plasma obtained from blood taken on day 2 (51 h) from *P. falciparum* infected patients treated with three tablets of Fansidar™ (1500 mg sulfadoxine–75 mg pyrimethamine). Peak 1: sulfadoxine, Peak 2: *N*-acetyl sulfadoxine, IS: sulfamethoxazole (internal standard).

at a flow rate of 1.0 ml min^{-1} on a LiChrospher™ RP-18 reversed-phase column. Such a modification gave base line resolution of sulfadoxine, *N*-acetyl sulfadoxine and the internal standard. No appreciable change in the peak area of sulfamethoxazole (internal standard; 50 μg ml^{-1}) was observed after 40 plasma extracted samples reinjections and the values were within ±8.2% of the mean value. Five samples each of plasma extracted samples of sulfadoxine (50 μg ml^{-1}) and *N*-acetyl sulfadoxine (50 μg ml^{-1}) were also evaluated and the values were within ±6.5% of the mean value. Fig. 2a shows the chromatographic behavior of a blank plasma extract from a healthy volunteer before administration of the drug while Fig. 2b represents the chromatogram of a plasma extract obtained from blood taken on day 2 (51 h) from a *P. falciparum* infected patient treated with three tablets of Fansidar™.

Table 1
Capacity factor[a] of various antimalarial drugs

Antimalarials	k'
Sulfadoxine	4.60
Sulfamethoxazole (Internal standard)	5.24
N-Acetyl sulfadoxine	7.42
Sulfalene	3.44
Pyrimethamine	13.44
Quinine	3.00
Amodiaquine	4.22
Chloroquine	4.46
Dapsone	3.28
Primaquine	7.02

[a] Mobile phase: acetonitrile–methanol–trifluoroacetic acid–water(16.1:7.2:0.1:76.6, v/v/v/v); flow rate 1.0 ml min^{-1}; UV detection 254 nm; column: LiChrospher™ RP-18 (5 μm, 250 × 4 mm).

Table 2a
Accuracy and precision of the HPLC method ($n = 10$) for the determination of sulfadoxine in spiked plasma samples

Concentration added ($\mu g\,ml^{-1}$)	Concentration found ($\mu g\,ml^{-1}$)	Error (%)	Accuracy (%)	RSD (%)
Intra-day				
2.5 (LLQ)	2.4	5.2	94.8	3.8
10	9.7	3.4	96.6	1.7
25	23.6	5.6	94.4	3.9
50	47.6	4.9	95.1	2.1
100	96.7	3.3	96.7	1.4
Mean ± SD			95.5 ± 1.1	2.6 ± 1.2
Internal standard 50 ($\mu g\,ml^{-1}$)	48.0	4.0	96.0	1.5
Inter-day				
2.5 (LLQ)	2.3	7.2	92.8	4.5
10	9.6	3.6	96.4	1.8
25	23.3	6.9	93.1	2.8
50	47.2	5.7	94.3	1.8
100	95.6	4.4	95.6	1.3
Mean ± SD			94.4 ± 1.6	2.4 ± 1.3
Internal standard 50 ($\mu g\,ml^{-1}$)	48.1	3.8	96.2	0.9

The capacity factors (k′) of most common antimalarial drugs detected by UV (254 nm) are given in Table 1, which shows that sulfalene, amodiaquine, dapsone, quinine, primaquine, pyrimethamine and chloroquine do not interfere in the determination of sulfadoxine and *N*-acetyl sulfadoxine by this method. Fifteen plasma samples from drug-free volunteers were extracted and analyzed. Interference due to endogenous compounds was not detected. The lower limit of detection for sulfadoxine and *N*-acetyl sulfadoxine was 0.01 $\mu g\,ml^{-1}$ and the limit of quantification was 2.5 $\mu g\,ml^{-1}$ with a mean intra-assay precision (RSD) of 3.8% ($n = 10$) with an accuracy of 94.8%.

Table 2b
Accuracy and precision of the HPLC method ($n = 10$) for the determination of *N*-acetyl sulfadoxine in spiked plasma samples

Concentration added ($\mu g\,ml^{-1}$)	Concentration found ($\mu g\,ml^{-1}$)	Error (%)	Accuracy (%)	RSD (%)
Intra-day				
2.5 (LLQ)	2.4	6.0	94.0	2.5
10	9.8	2.4	97.6	3.3
25	23.9	4.6	95.4	4.0
50	47.6	4.8	95.2	1.9
100	96.6	3.4	96.6	2.1
Mean ± SD			95.8 ± 1.4	2.8 ± 0.9
Inter-day				
2.5 (LLQ)	2.3	6.8	93.2	3.4
10	9.7	3.0	97.0	3.8
25	23.8	5.0	95.0	2.8
50	47.6	4.8	95.2	2.1
100	96.4	3.6	96.4	1.8
Mean ± SD			95.4 ± 1.5	2.8 ± 0.8

Table 3
Extraction recovery of the HPLC method ($n = 10$) for sulfadoxine, *N*-acetyl sulfadoxine and sulfamethoxazole (IS) in plasma

Concentration ($\mu g\,ml^{-1}$)	Recovery (%) mean ± SD		
	Sulfadoxine	*N*-Acetyl sulfadoxine	Sulfamethoxazole
2.5 (LLQ)	86.8 ± 4.2	82.9 ± 2.5	
10	93.2 ± 3.0	85.6 ± 6.6	
25	88.5 ± 5.3	86.6 ± 4.0	
50	92.6 ± 3.0	87.9 ± 1.9	92.3 ± 2.5
100	92.0 ± 3.8	91.6 ± 4.0	
Mean ± SD	90.6 ± 2.8	86.9 ± 3.2	

The average calibration equation relating y (drug/IS; peak area ratio) to × (concentration $\mu g\,ml^{-1}$) calculated from 10 analytical runs at 5 concentrations (range 2.5–100 $\mu g\,ml^{-1}$) based on therapeutic values reported earlier [13,19] were as follows: (i) sulfadoxine, $y = 0.314x + 0.696$; (ii) *N*-acetyl sulfadoxine, $y = 1.048x - 0.729$. The coefficient of determination (r^2) was always >0.999.

Intra-day mean relative standard deviations (RSD's) for sulfadoxine and *N*-acetyl sulfadoxine were 2.6 and 2.8%, respectively while inter-day mean RSD's for sulfadoxine and *N*-acetyl sulfadoxine were 2.4 and 2.8%, respectively (Tables 2a and 2b). Intra-day assay RSD's for sulfadoxine were ≤3.9% and for *N*-acetyl sulfadoxine ≤4.0% while inter-day assay RSD's were ≤4.5% for sulfadoxine and ≤3.8% for *N*-acetyl sulfadoxine. Mean accuracy for sulfadoxine and *N*-acetyl sulfadoxine was ≥92.8% (Tables 2a and 2b). Extraction recoveries averaged 90.6% for sulfadoxine and 86.9% for *N*-acetyl sulfadoxine. The average recovery of sulfamethoxazole was 92.3% (Table 3). Extraction recovery of sulfadoxine from human plasma was similar to that reported earlier [13,19].

Sulfadoxine, *N*-acetyl sulfadoxine and internal standard were tested for short and long-term stability stored in methanol or plasma. There was no significant loss in the concentrations of sulfadoxine, *N*-acetyl sulfadoxine or internal standard and coefficient of variations confers stability of these compounds during analysis and experimentation. The freeze–thaw cycle did not reflect a major variation of the concentrations of the analytes and the variation was within acceptable limits.

Mean parasite density from 52 *P. falciparum* cases was 11,360 parasites μl^{-1} blood (ranged from 8380 to 14,236 parasites μl^{-1} blood). In 48 *P. falciparum* cases, parasites cleared within 7 days of treatment (classified as sensitive) while in 4 cases, parasites reappeared within 10–20 days (classified as resistant). All four cases were treated with artesunate and responded well. Mean plasma concentrations of sulfadoxine and *N*-acetyl sulfadoxine from the 48 sensitive *P. falciparum* cases and those in 4 resistant cases on day 2 (51 h) after administration of 3 tablets of Fansidar™ are given in Table 4. The mean sulfadoxine concentration in the plasma samples collected from the sensitive cases was higher than those in the samples from

Table 4
Sulfadoxine and *N*-acetyl sulfadoxine concentration in plasma of sensitive and resistant *P. falciparum* malaria cases

Drugs	Mean concentration ($\mu g\,ml^{-1}$)[a] ± SD	
	Sensitive mean ($n=48$)	Resistant ($n=4$)
Sulfadoxine	62.8 ± 12.9	60.5 ± 6.0
N-Acetyl sulfadoxine	5.7 ± 1.7	8.4 ± 0.4
N-Acetyl sulfadoxine/sulfadoxine (%)	9.1	13.9

[a] Concentrations on day 2 (51 h) after administration of three tablets of Fansidar™.

the resistant patients; however, the difference was statistically insignificant (62.8 $\mu g\,ml^{-1}$ vs. 60.5 $\mu g\,ml^{-1}$; $P > 0.05$). Dzinjalamala et al. [17] showed a trend towards lower sulfadoxine levels in patients with treatments failure than those with adequate clinical and parasitological response. The mean ratio of *N*-acetyl sulfadoxine to sulfadoxine was 9.1% in responders (sensitive) and 13.9% in non-responders (resistant) and found significantly different ($P < 0.01$) using student's *t*-test. Sarikabhuti et al. [14] found significantly higher amount of *N*-acetyl sulfadoxine in plasma of non-responders. Carmona et al. [20] reported that the median blood concentration of sulfadoxine 2 h after dosing in patients with good clinical response and non-responders were 42.3 and 32.1 $\mu g\,ml^{-1}$, respectively. The inter-individual variation of sulfadoxine in plasma was 2.4-fold while that of *N*-acetyl sulfadoxine was 3.5-fold which is similar to earlier reports [14,19].

4. Conclusions

The HPLC method described here for the determination of sulfadoxine and its metabolite *N*-acetyl sulfadoxine in human plasma from *P. falciparum* malaria patients is appropriately sensitive, selective and reproducible. This is the first HPLC method developed specifically for the analysis of sulfadoxine and *N*-acetyl sulfadoxine in plasma from malaria patients and should find an immediate application in the therapeutic monitoring of such patients. The ratio of the concentration of *N*-acetyl sulfadoxine to that of sulfadoxine may give a useful indication of the difference in response to sulfadoxine in sensitive and resistant *P. falciparum*.

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